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SHORT COMMUNICATION

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We report the selection and characterization of influenza A/NWS-G70c and B/HK/8/73 (HG) viruses which are resistant to the potent influenza neuraminidase inhibitor, 4-guanidino-Neu5Ac2en. Viruses were selected which replicated in MDCK cells in the presence of 20 μg/ml inhibitor. The neuraminidase of resistant viruses was >200-fold more resistant to 4-guanidino-Neu5Ac2en than was the neuraminidase of the parent viruses. Although amounts of neuraminidase protein were similar in resistant and parent viruses, the enzyme activity of the resistant neuraminidase heads was reduced by >95% for the substrates used. Relative to parent viruses, the resistant viruses replicated to equal or greater titers in tissue culture and in embryonated chicken eggs. Sequence analysis revealed a single nucleotide mutation in the neuraminidase gene of each virus resulting in the change of the conserved Glu 119 (which lies in a pocket beneath the active site of the enzyme) to Gly thus eliminating an electrostatic interaction with the C-4 guanidinium moiety of the inhibitor. Mutations (Asn → Ser) at amino acids 145 and 150 were also found in the hemagglutinin gene of the B/HK/8/73 (HG) virus resistant to 4-guanidino-Neu5Ac2en. No changes were found in the hemagglutinin gene of the resistant A/NWS-G70c virus.

Influenza viruses encode two envelope glycoproteins, neuraminidase (NA) and hemagglutinin (HA), for which high resolution crystal structures are available (*1, 2*). HA mediates attachment of the virus to the host cell and fusion with the endosomal membrane. NA (EC 3.2.1.18; sialidase, neuraminidase, acylneuraminylhydrolase) catalyzes the removal of terminal sialic acid residues from viral and cellular glycoconjugates and facilitates the release of virus from the infected cell.

The catalytic sites of the neuraminidases from all influenza A and B viruses so far examined are structurally very similar (*3, 4*) and several amino acids which line the active site of the enzyme and participate directly in the binding and catalysis of the substrate are invariant (reviewed in *5*). This suggests that an active-site inhibitor effective against one strain should, in principle, be effective against all strains of influenza A and B viruses. A structure-based approach to the design of influenza neuraminidase inhibitors led to the discovery that the sialic acid analog, 4-guanidino-Neu5Ac2en (4-GuDANA), is a potent inhibitor of the enzyme as well as influenza virus replication (*6–8*). This inhibitor binds in the catalytic site of influenza virus NA and it has been shown that the C-4 guanidinium moiety of the inhibitor forms a strong electrostatic interaction with the conserved glutamic acids at amino acid positions 119 and 227 (*6, 9*).

Here, the selection of influenza A and B viruses for resistance to 4-GuDANA is reported. 4-Guanidino-2-deoxy-2,3-didehydro-D-*N*-acetylneuraminic acid (4-GuDANA) was synthesized according to published procedures with modifications (*10, 11*). The reassortant influenza viruses A/NWS/33_{HA}-A/Tern/Australia/G70c/75_{NA}, referred to as A/NWS-G70c (*12*), and the high-titer growing influenza B/HK/8/73 (HG), which has the HA of B/Hong Kong/8/73 and the NA of B/Lee/40 (*13*), were grown in Madin-Darby canine kidney (MDCK) cells in step-wise increases of 4-GuDANA up to a concentration of 20 μg/ml and plaque purified three times in 5 to 10 μg/ml of inhibitor. The 4-GuDANA resistant (4-GuDANA^r) viruses, unlike parent viruses, were able to replicate and induce cytopathic effects in MDCK cells in the presence of 5 μg/ml 4-GuDANA (Fig. 1). In additional studies, using a standard plaque assay (*14*) performed in triplicate to estimate 50% inhibitory end-points (*15*), the concentration of 4-GuDANA required to reduce the number of plaques by 50% was 0.2 μg/ml for A/NWS-G70c, 4.6 μg/ml for 4-GuDANA^r A/NWS-G70c, 0.07 μg/ml for B/HK/8/73 (HG), and 6.0 μg/ml for 4-GuDANA^r B/HK/8/73 (HG).

The activity of virion associated or purified neuraminidases from parent or 4-GuDANA^r influenza A and B viruses was assayed using either fetuin (*16*) or 2′-(4-meth-

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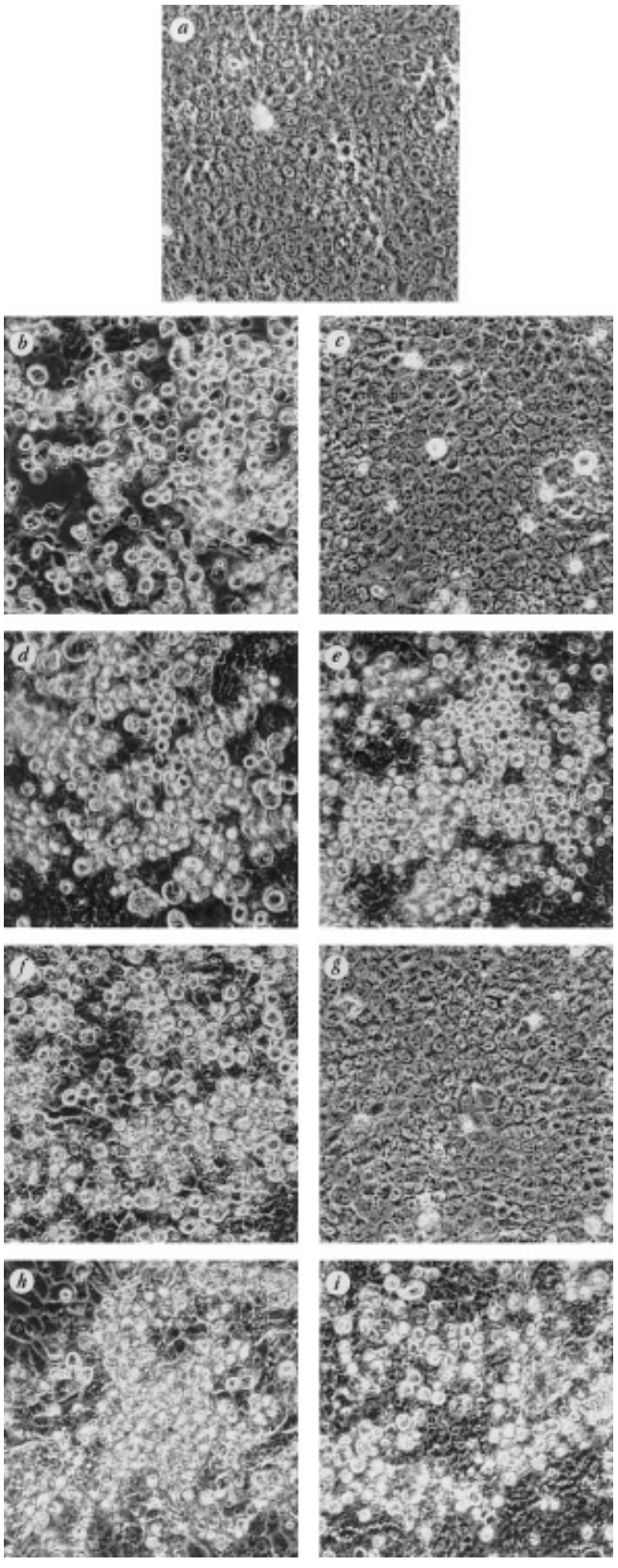

FIG. 1. Effect of 4-GuDANA on the replication of parent and 4-GuDANA^r influenza A and B viruses. MDCK cells were infected with the parent viruses, A/NWS-G70c or B/HK/8/73 (HG) or the 4-GuDANA^r viruses derived from these and incubated in growth medium with or without 5 μg/ml of 4-GuDANA at 37°. After 72 hr, phase-contrast photomicrographs (400X) of the cell monolayers were taken. (A) mock infected. (B) A/NWS-G70c infected and untreated. (C) A/NWS-G70c treated with 4-GuDANA. (D) 4-GuDANA^r A/NWS-G70c infected and untreated. (E) 4-GuDANA^r A/NWS-G70c infected and treated with 4-GuDANA. (F) B/HK/8/73 (HG) infected and untreated. (G) B/HK/8/73 (HG) infected and treated with 4-GuDANA. (H) 4-GuDANA^r B/HK/8/73 (HG) infected and untreated. (I) 4-GuDANA^r B/HK/8/73 (HG) infected and treated with 4-GuDANA.

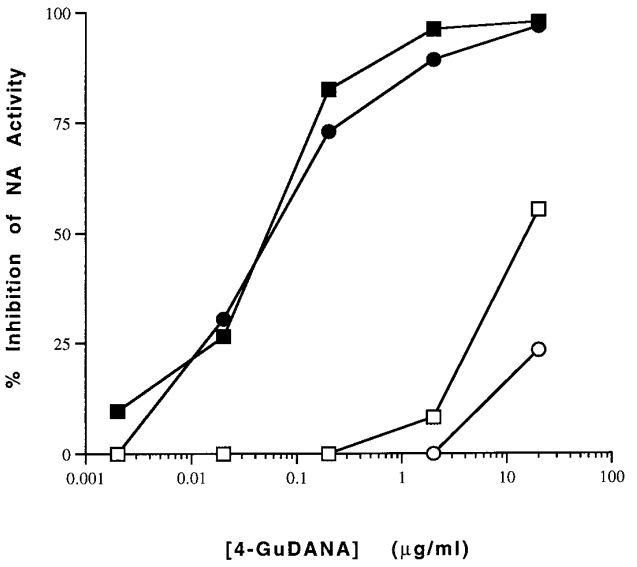

FIG. 2. Inhibition of virion-associated NA by 4-GuDANA. Using fetuin as the substrate (*16*), NA activity associated with A/NWS-G70c (■), 4-GuDANA^r A/NWS-G70c (□), B/HK/8/73 (HG) (●), or 4-GuDANA^r B/HK/8/73 (HG) (○) virions was assayed in the absence or presence of increasing concentrations of 4-GuDANA. The concentration of 4-GuDANA required to inhibit neuraminidase activity by 50% (*15*) was 0.05 μg/ml for A/NWS-G70c, 15.5 μg/ml for 4-GuDANA^r A/NWS-G70c, 0.10 μg/ml for B/HK/8/73 (HG), and $>$20 μg/ml for 4-GuDANA^r B/HK/8/73 (HG). Similar results were obtained using NA heads purified from resistant and parent viruses (data not shown).

ylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUN) (*17*) as the substrate. The NA activity associated with 4-GuDANA^r virions, as determined using fetuin as the substrate, was $>$200-fold more resistant to inhibition by 4-GuDANA than was enzyme activity associated with parent viruses (Fig. 2). The NA activity of 4-GuDANA^r influenza A and B viruses was also resistant to 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA; data not shown).

NA heads were prepared by protease digestion of virus particles obtained from infected 11-day-old embryonated chicken eggs and purified by sucrose gradient centrifugation as previously described (*12, 18*). When normalized for protein content, purified NA heads from 4-GuDANA^r influenza A or B viruses displayed much less NA activity for both fetuin and MUN substrates when compared to NA heads from parent viruses (Table 1). The decrease in enzyme activity was not due to a decrease in the NA protein content of the resistant viruses since the

TABLE 1

Relative Activities of NA Purified from Parent and 4-GuDANA^r Influenza A and B Viruses

Virus[a]	MUN[b]	Fetuin
A/NWS-G70c	100%[c]	100%
4-GuDANA^r A/NWS-G70c	4.9%	5.0%
B/HK/8/73 (HG)	100%	ND[d]
4-GuDANA^r B/HK/8/73 (HG)	0.6%	ND

[a] Virus was obtained from the allantoic fluid of 11-day embryonated chickens eggs and purified by adsorption to and elution from chicken red blood cells followed by sucrose gradient centrifugation as previously described (*18*). NA heads were obtained by protease digestion of purified virus particles (*12*).

[b] Fetuin and the fluorogenic subtrate, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUN), were purchased from Sigma Chemical Co. (St. Louis, MO). The activity of purified neuraminidases from parent and 4-GuDANA^r influenza A and B viruses was assayed using either fetuin (*16*) or MUN (*17*) as the substrate and was normalized to neuraminidase protein content.

[c] The level of NA activity for each of the parent A and B influenza virus enzymes was set to 100% and the level of enzymatic activity of the 4-GuDANA^r neuraminidases is relative to that of the respective parent enzyme. Similar results were obtained using virions as the source of NA (data not shown).

[d] Not determined.

yield of purified NA from resistant and parent viruses was similar (data not shown). Although the 4-GuDANA^r viruses were found to have markedly lower levels of NA activity than did parent viruses, the resistant viruses replicated to high titers in embryonated eggs (data not shown). In MDCK cells, 4-GuDANA^r influenza A and B viruses were not impaired for multicycle replication. The resistant A/NWS-G70c virus replicated to peak titers which were equivalent to those of the parent virus, although this virus displayed delayed kinetics reaching a maximum titer 12 hr later than did the parent virus (Fig. 3). The resistant B/HK/8/73 (HG) virus displayed normal replication kinetics and grew to higher titers than did the parent virus (Fig. 3). The results here indicate that the greatly reduced levels of NA activity associated with 4-GuDANA^r viruses are sufficient to permit multiple cycles of replication in tissue culture and *in ovo.* Along these lines, an A/WSN/33 virus containing an altered NA displayed only approximately 10% of the wild-type NA activity yet was still capable of multiple rounds of replication in MDCK cells (*19*). However, the complete lack of NA results in the aggregation of virus particles at the infected cell surface and the inability to undergo multicycle replication (*20*).

Influenza A and B viruses were obtained from 11-day-old embryonated chicken eggs and purified on sucrose gradients, as was done for the preparation of NA heads, and were used as the source of viral RNA for sequence analysis. The complete nucleotide sequences of NA and HA genes from parent and from 4-GuDANA^r A/NWS-G70c and B/HK/8/73 (HG) viruses were determined using synthetic oligonucleotide primers according to standard procedures (*21, 22*). The NA gene of the 4-GuDANA^r A/NWS-G70c (N9) virus was found to have a single mutation leading to the amino acid substitution, Glu → Gly at position 119 (N2 numbering). Similarly, the NA gene of the 4-GuDANA^r B/HK/8/73 (HG) also had a single mutation leading to the amino acid substitution Glu → Gly at position 119. This glutamic acid lies in a pocket beneath the active site of the enzyme (*2, 6, 9*) and is absolutely conserved among all known influenza virus neuraminidases (Fig. 4). Our data provide genetic evidence in support of recent structural findings which show a strong electrostatic interaction between the carboxylate of Glu 119 and the C-4 guanidinium group of 4-GuDANA (*6, 9*). The replacement of Glu 119 with Gly in viruses which are resistant to 4-GuDANA underscores the importance of this interaction as the basis for the inhibitory activity of this sialic acid analog.

There were no differences between the HA gene of the parent A/NWS-G70c virus and that of the 4-GuDANA^r mutant. In contrast, two mutations were observed in the HA gene from 4-GuDANA^r B/Hong Kong/8/73 (HG); one at nucleotide position 489 and the other at nucleotide position 504, both resulting in a codon change of AAC → AGC yielding changes of Asn → Ser at amino acids 145 and 150. This suggests that, at least for this type B virus, alterations in the HA, in response to the selective pressure of 4-GuDANA, may play a role in the resistance to this inhibitor. Since Asn 145 is part of a Asn-X-Ser/

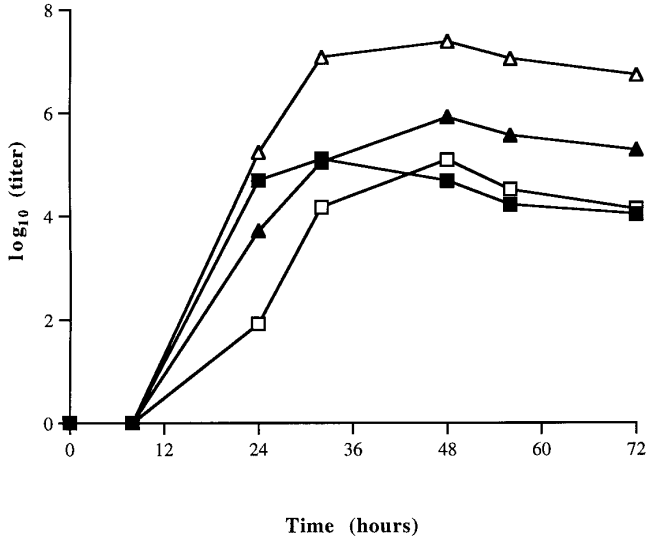

FIG. 3. Replication of parent and 4-GuDANA^r influenza A and B viruses in MDCK cells. MDCK cells were infected with A/NWS-G70c (■), 4-GuDANA^r A/NWS-G70c (□), B/HK/8/73 (HG) (▲), or 4-GuDANA$_r$ B/HK/8/73 (HG) (△) at a multiplicity of 0.001 and the infected cells were incubated in medium without 4-GuDANA. At the indicated times postinfection, cell culture fluid was harvested and the virus titer was determined in MDCK cells using a standard plaque assay in the absence of 4-GuDANA (*14*).

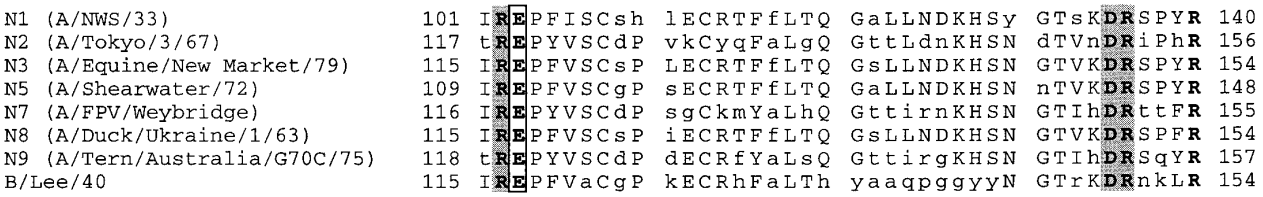

FIG. 4. Alignment of the amino acid sequences of different influenza virus neuraminidases in the region surrounding Glu 119 (N2 numbering). Conserved amino acids are indicated by upper case letters. The conserved Glu which is changed to Gly in the 4-GuDANA^r mutants is shown in bold type and boxed. Active site amino acids which directly contact sialic acid are shaded. Second shell amino acids are shown in bold type. Sequence comparison of the NA genes from parent A/NWS-G70c and B/HK/8/73 (HG) and the 4-GuDANA^r viruses revealed a single substitution at nucleotide position 377 (A virus) or 403 (B virus) resulting in a codon change of GAA → GGA yielding a change at amino acid position 120 (N9 numbering) or 117 (B/Lee/40 numbering) of Glu → Gly.

Thr consensus sequence for N-linked glycosylation, the change of this amino acid to Ser would eliminate this glycosylation site. Because of the change from Asn → Ser at amino acid 150, Asn 148 becomes a potential site for the attachment of a glycosyl group. If this is indeed the case, the new location of the carbohydrate moiety (at Asn 148) in the HA of 4-GuDANA^r B/HK/8/73 (HG) virus could conceivably affect the availability and/or the affinity of this envelope glycoprotein for its sialic acid receptor. Alternatively, alterations in the glycosylation pattern of HA might affect the cleavability of HA by host cell protease(s), a step which is essential for entry of influenza virus into the host cell (*23*). Because of the role of NA in the cleavage of HA by cellular protease(s) (*19, 24*), the alteration in HA of the 4-GuDANA^r virus might be a compensatory response to the greatly reduced NA levels associated with this virus. The HA genes from other strains of influenza A and B viruses selected for resistance to 4-GuDANA will have to be sequenced in order to determine the general relevance of these results.

The determination of whether the Glu → Gly change found in the NA of 4-GuDANA^r influenza viruses is sufficient to confer resistance to this inhibitor will require the introduction of the necessary mutation into the NA gene of influenza viruses using the technique of reverse genetics (*25*). Furthermore, the introduction of the HA mutations reported here into HA gene of influenza B virus, in the absence of any changes in the NA gene, would elucidate the contribution, if any, of these changes to the 4-GuDANA^r phenotype. Experiments along these lines are in progress.

The two viruses used for the selection of mutants resistant to 4-GuDANA were chosen because each possesses NA proteins which readily form large, well-ordered crystals. Both N9 and B/Lee/40 NA crystals diffract X rays to high resolution and the structure of each enzyme has been determined (*3, 26*). Under identical conditions, mutant N9 NA formed crystals having similar morphology to those of the enzyme from the parent virus but none grew to a size larger than 0.2 mm in diameter, in contrast to the parent N9 which readily formed crystals which grew to 1.0 mm or greater. Attempts will be made to collect X-ray diffraction data from these crystals. The mutant B/Lee/40 NA has so far produced only very small irregular crystals, not suitable for analysis by x-ray diffraction, in contrast to the parent B/Lee/40 enzyme which formed crystals of high quality. These results suggest that the sequence change of Glu 119 → Gly affects not only the enzyme activity of the NA but also the surface properties of the enzyme. The exact structural changes involved will not be fully appreciated until X-ray diffraction quality crystals of the mutant enzymes can be grown.

The clinical relevance of the work reported here remains to be determined and two issues need to be addressed: (1) It would be of great interest to determine whether influenza viruses which are resistant to 4-GuDANA display altered pathogenicity and virulence in an animal model of infection. (2) Although influenza A and B viruses resistant to 4-GuDANA were isolated readily in tissue culture, the experiments described here do not provide an estimate of the frequency at which the drug-resistant mutants can be isolated in a population of viruses. In preliminary experiments in animals treated with this inhibitor, the appearance of influenza virus resistant to 4-GuDANA could not be demonstrated (*6*). In a clinical study of experimental influenza virus infection, 4-GuDANA demonstrated therapeutic efficacy against influenza A/Texas/91 (H1N1) infection but the emergence of resistant virus was not reported (*27*).

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